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13. ABSTRACT ***(Maximum 200 Words)***
tGolgin-1 is a large peripheral membrane protein that consitsts predominantly of coiled coil regions and associates with the *trans* Golgi network (TGN) by virtue of a C-terminal GRIP domain. Our work has shown that overexpression of isolated GRIP domains from tGolgin-1 or a related protein, golgin-97, results in disruption of the structure, protein content, and function of the TGN, suggesting that tGolgin-1 and other GRIP domain proteins function in regulating TGN structure and function. This is important because signalling molecules required for initiating transformation and for metastasis rely on sorting and processing in the TGN. We have established tools to dissect the molecular basis of GRIP domain function and of the predicted N-terminal domain. More recently we have successfully used RNAi to eliminate expression of tGolgin-1 from mammalian cells. The results suggest that tGolgin-1 may play a more broad role in positioning the Golgi complex at the microtubule organizing center of the cell. This has important implications for a role of tGolgin-1 in cell motility required both for tumor metastasis and for the targeting of tumors by inflammatory cells and cytotoxic T lymphocytes.

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INTRODUCTION

This report summarizes the training and research accomplishments over the first year of funding from award # DAMB17-01-1-0365. The award supports research directed toward understanding the function of tGolgin-1 (also known as golgin-245, trans golgi p230, and 256 kD golgin), a peripheral membrane protein associated with the *trans* Golgi network (TGN) of mammalian cells(Erlich et al., 1996; Fritzler et al., 1995; Gleeson et al., 1996). tGolgin-1 is a member of a family of large peripheral membrane proteins that are predicted to consist largely of coiled-coil structure with a small predicted globular region of homology at the C-terminus referred to as the GRIP domain. The GRIP domain was shown by several groups to mediate localization of these proteins to the cytosolic face of the Golgi complex and/or TGN(Barr, 1999; Brown et al., 2001; Kjer-Nielsen et al., 1999; Munro and Nichols, 1999). A function for tGolgin-1 or any of the other GRIP domain proteins has not yet been described.

BODY

Our preliminary data suggested that GRIP domain proteins contribute to the integrity of the TGN and to protein sorting and processing events that occur within the TGN. We showed that overexpression of isolated, epitope-tagged GRIP domains from either tGolgin-1 or golgin-97 resulted in displacement of endogenous GRIP domain proteins from the Golgi complex. This displacement was associated with defects in resident integral membrane protein localization to the TGN protein as well as TGN structure and function. Among the functions disrupted by GRIP domain overexpression were (1) the cell surface delivery of at least a subset of integral membrane proteins and (2) the cleavage of substrate proproteins by proprotein convertases of the furin family. Among those proteins that require tGolgin-1 for cell surface delivery are likely growth factor receptors associated with breast cancer induction, such as epidermal growth factor receptor and Her2. Proprotein convertase substrates include matrix metalloproteinases required for metastasis of breast tumors. Thus, the effects GRIP domain overexpression may influence the growth and metastasis of tumors; moreover, dissecting how GRIP domain protein structure and function are related may reveal novel approaches for treatment of breast tumors. The goals of the experiments as proposed in the *Statement of Work* were to elucidate such structure/function relationships by dissecting functional domains of tGolgin-1 and by characterizing their interacting partners.

Since the inception of the funding from this grant, we have made significant progress in shoring up our preliminary data, advancing our abilities to tackle the originally proposed aims in the *Statement of Work*, and progressing in some new and very exciting directions. In the context of these advances and recently published work from other groups, the aims in the *Statement of Work* have been somewhat modified. Below we detail our progress.

1. <u>Sequence and expression of mouse tGolgin-1</u>. We have completed an initial characterization of the mouse tGolgin-1 cDNA and the expression of the corresponding mRNA in different tissues. In collaboration with Drs. D. Gay and M. I. Greene, we have shown that tGolgin-1 expression is highly but transiently upregulated during oligodendrocyte development. These findings are presented in a manuscript currently in press in DNA and Cell Biology (Cowan, D*et al.*, 2002. <u>DNA and Cell Biol.</u>, in press; **see appendix for galley proof of manuscript**). Dr. Yoshino is a co-author of this work.

2. <u>Role of GRIP domain proteins in maintenance of the TGN.</u> We have completed the work on this study and a manuscript is currently in preparation. Dr. Yoshino is the first author on this work. The main findings of the study are that overexpression of GRIP domains results in (1) displacement of endogenous TGN resident proteins, but not residents of the Golgi stack or other subcellular organelles, to peripheral membranes, including multivesicular late endosomes; (2) vacuolization of the Golgi and the TGN; (3) disruption of protein transport of the vesicular stomatitis virus G protein from the Golgi to the plasma membrane; and (4) disruption of furin-dependent cleavage of a substrate protein. We speculate that this implicates a role for GRIP domain proteins or GRIP domain ligands in regulating the recycling of TGN resident proteins from endosomal compartments back to the TGN. We hope to submit this manuscript to Mol. Biol. Cell by mid-August. **See appendix for an abstract of this work.**

3. Molecular determinants of tGolgin-1 function. This was the main thrust of the work proposed in the *Statement of Work*. Some of the aims have been reprioritized and others have been changed based on published work and unpublished communications from our collaborators.

Task 1 was to characterize membrane complexes containing endogenous tGolgin-1 or its derivative N-terminal or GRIP domains. While this still remains a goal of the study, it is no longer the primary goal. Furthermore, much of the proposed experiments were geared toward understanding how GRIP domain localization was mediated by small GTPases of the rab superfamily; this was based on published evidence that (1) Rab6 bound to GRIP domains of tGolgin-1 and golgin-97(Barr, 1999) and (2) GTPγS treatment prevented release of full-length tGolgin-1 or its isolated GRIP domain from the Golgi region of permeabilized cells(Brown et al., 2001; Gleeson et al., 1996). However, the published evidence for the Rab6 interaction was weak. Furthermore, our work in which GRIP domain were coexpressed with dominant-acting mutants of Rab6 raised questions concerning the physiological relevance of a Rab6-GRIP domain interaction. We thus interpret these data to indicate that a different GTP-binding protein may be important for GRIP interactions with Golgi membranes. Over the last year, two groups published that GRIP domains interacted with members of a different family of small GTPases, the Arf-like proteins Arl1 and Arl3, by yeast two-hybrid analyses(Lu et al., 2001; Van Valkenburgh et al., 2001). Our collaborator, Dr. Chris Burd, has shown functional interactions of GRIP domains and Arl proteins in yeast (unpublished data). We have therefore generated a panel of reagents to dissect functional and physical interactions of Arl1 and Arl3 with GRIP domain *in vivo* and *in vitro*. We hope to complete these studies now in the next funding period, replacing the described studies in the *Statement of Work* in dissecting Rab6 interactions..

Task 2 and *Task 3* were to identify the requirements for tGolgin-1 binding to the TGN membrane via the GRIP domain, and to endosomal membranes via the N-terminal domain, respectively. We have now generated the required reagents (bacterially expressed GST fusion proteins and mammalian expression vectors for requisite fragments) and feel confident that we can proceed rapidly with these experiments. Instead of Rab proteins as initially proposed, we have generated the reagents to test for a role for Arl1 and Arl3 in these activities, as described above. We have performed intial analyses of GRIP domain binding to phosphatidylinositides via a simplified dot-blotting procedure, but thus far have not observed any interactions. We are preparing to study interactions with a more broad spectrum of lipids using the recombinant fragments that we have generated.

In brief, we have reprioritized several of the aims such that *Task 2* and *Task 3* were given priority over *Task 1*. These tasks required the generation of a number of reagents which are now in hand. We should be able to succeed in these aims very rapidly in the coming year.

4. Functional analysis of tGolgin-1. These studies were initiated earlier this year as the result of emerging technology using inhibitory RNAs (RNAi). Recent work in mammalian cultured cells has shown that transfection with small duplexes of RNAs (19-21 bp) with short overhanging ends can result in the selective elimination of the corresponding complementary mRNA(Elbashir et al., 2001; Elbashir et al., 2002). These small inhibitory RNAs (siRNA) can therefore be used essentially to functinally "knock-out" a gene of interest in cultured cells. We generated phosphoramidite substituted oligoribonucleotides corresponding to part of the coding region near the 5' end of the tGolgin-1 sequence and to part of the 3' untranslated region. These putative siRNAs were expressed by transfection in cultured cells, and expression of endogenous tGolgin-1 was monitored by immunofluorescence microscopy and/or western blotting. Using this protocol, we were able to eliminate detectable expression of tGolgin-1 in 50-90% of transfected cells. Analyses of these cells by immunofluorescence microscopy showed that markers of the TGN were dislocated to peripheral punctate structures (**Fig. 1**). However, unlike in cells overexpressing GRIP domains, some markers of the Golgi stack also appear to be dislocated. We are currently further assessing the nature of these peripheral structures and the localization of markers for other organelles. These studies should clearly identify protein transport steps in which tGolgin-1 functions. The completion of this work will be a major focus of Dr. Yoshino's continued studies.

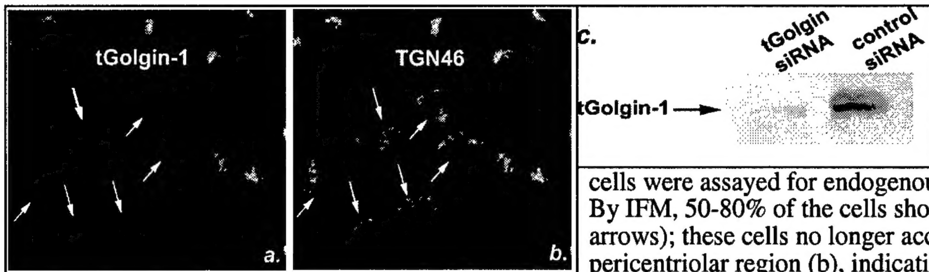

Figure 1. **siRNA-mediated down-regulation of tGolgin-1 in cultured cells.** HeLa cells were transfected with duplexes of 21bp oligoribonucleotides corresponding to nt 101-122 of the tGolgin-1 coding sequence (a-c) or a control (to Tsg101; c). Two days later cells were assayed for endogenous tGolgin-1 by IFM (a) or western blotting (c). By IFM, 50-80% of the cells showed undetectable reactivity for tGolgin-1 (a; arrows); these cells no longer accumulate the TGN marker, TGN46, near the pericentriolar region (b), indicating a defect in TGN localization or clustering. By enhancing transfection efficiencies to 80-90%, reduced steady state levels of tGolgin-1 could be observed by western blotting (c).

Additional Training

Dr. Yoshino has made great strides in developing as an independent researcher over the last year. She developed Aim 4 above nearly completely independently of Drs. Marks and Lemmon, and has made significant advances in a short period of time. Dr. Yoshino is now gaining experience supervising technical staff and expanding her field of work. She is learning new biochemical approaches for all of the aims and will be well armed with techniques for future endevors. She has also dramatically improved in her presentation skills, as evident from group meetings of the Marks and Lemmon labs with those of Drs. Chris Burd and Margaret Chou as well as in small group meetings of the Marks lab alone. Dr. Yoshino also presented her work at a meeting of the Japanese Biochemical Society in October, 2001 (**see appendix for abstract of this work**), and plans to attend this meeting again in 2002.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of sequencing and expression of mouse tGolgin-1 (manuscript in press)
- Completion of analysis of GRIP domain overexpression (manuscript in preparation)
  - mislocalization of TGN resident integral membrane proteins
  - vacuolization of the TGN
  - disruption of TGN function (sorting to plasma membrane and furin-dependent proprotein processing)
- Development of all necessary reagents for project described in *Statement of Work*
- Functional analysis of tGolgin-1 using siRNA
  - dislocation of TGN resident integral membrane proteins
  - dislocation of Golgi resident proteins?

REPORTABLE OUTCOMES

1. Manuscript in press: D. Cowan, D. Gay, B.M. Bieler, H. Zhao, **A. Yoshino**, J.G. Davis, M.M. Tomayko, R. Murali, M.I. Greene and M.S. Marks (2002). Characterization of mouse tGolgin-1 (golgin-245/ trans golgi p230/ 256kD golgin) and its upregulation during oligodendrocyte development. <u>DNA and Cell Biol.</u> **21**: 505-517 (in press).

2. Manuscript in preparation: **A. Yoshino**, B.M. Bieler, D.C. Harper, D. Cowan, D. Gay, J.M. McCaffery and M.S. Marks. Regulation of TGN protein localization by GRIP domain proteins. In preparation.

3. Abstract for Meeting of the Japanese Biochemical Society: **A. Yoshino**, B.M. Bieler, D.C. Harper, D. Cowan, D. Gay, J.M. McCaffery and M.S. Marks. Regulation of TGN protein localization by GRIP domain proteins.

CONCLUSIONS

Our research thus far has assigned a function to tGolgin-1 and potentially other GRIP domain proteins, a group of peripheral Golgi-associated proteins that have been described for a number of years but for which no function has been known. These proteins appear to be involved in a critical step of protein transport to or from the TGN, a subcellular organelle required for protein sorting and modification within both the secretory and endocytic pathways. Based on our results in which GRIP domains were overexpressed in cells, we hypothesize that GRIP domain proteins or ligands of the GRIP domain function in vesicular transport from endosomal compartments to the TGN. This is a pathway that is required to maintain resident proteins within the TGN, and thus TGN function – such as delivery of critical growth factor receptors and proprotein cleavage of matrix metalloproteinases and other critical metastatic factors. The gene encoding tGolgin-1 has been shown to be deleted in at least one lung carcinoma (Ishikawa et al., 1997), suggesting that manipulation of its function may be important for tumorigenesis. Continued work on the function and physical interactions of tGolgin-1 and other GRIP domain proteins may therefore provide us with insights into manipulation of tumors. More directly, elucidating the function of these mysterious peripheral membrane proteins will help us to understand the biology of intracellular protein transport.

As discussed above, future work will focus on the interactions of GRIP domain proteins with members of the Arl family of small GTPases rather than with Rab proteins. An additional useful avenue of research, as exemplified by our successes with siRNA for tGolgin-1, will be to further characterize the defects imposed by loss of function of tGolgin-1 and to characterize the phenotypes resulting from siRNA-mediated disruption of other GRIP domain proteins, such as golgin-97.

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APPENDICES

1. Galley proof for manuscript in press: Cowan , et al., 2002.
2. Abstract from Meeting of the Japanese Biochemical Society

Characterization of a TGN-localized peripheral membrane protein, tGolgin-1.

A Yoshino, BM Bieler, DC Harper, DA Cowan, N Cole, S Sutterwala, D Gay, JM McCaffery* and MS Marks. Dept. of Pathology and Laboratory Medicine, Univ. of Pennsylvania, Philadelphia, PA 19104 and *Dept. of Biology, Johns Hopkins Univ., Baltimore, MD 21218.

tGolgin-1 (also known as trans golgi p230) is a largely coiled-coil peripheral membrane protein of unknown function. Human tGolgin-1 localizes to the cytosolic face of the trans Golgi network (TGN) and associated vesicles by virtue of its C-terminal "GRIP" domain. Overexpression of GRIP-domain containing fragments of tGolgin-1 (C312) or the related golgin-97 (C179) has been shown to disrupt the normal perinuclear distribution of endogenous tGolgin-1. We show that in cells overexpressing GRIP domain fragments, other TGN resident proteins, such as the integral membrane proteins TGN46 and furin and the coat protein AP-1, are also displaced as observed by immunofluorescence microscopy. In contrast, the distribution of residents of other organelles was not affected. Overexpression of GRIP-domain fragment affected TGN function; both the transport of VSV-G protein from the Golgi to the cell surface and protein cleavage by TGN resident protease furin were slowed. From preliminary results by electron microscopy, C312-expression may disrupt TGN structure, resulting in vacuolization of the TGN. The effects of GRIP domain overexpression were eliminated by mutagenesis of a conserved tyrosine residue that is required for GRIP-dependent TGN localization. We suggest that the C-terminal GRIP domain fragments compete with endogenous proteins for a limiting GRIP domain binding site, and thereby disrupt the function of endogenous GRIP domain proteins such as tGolgin-1. If this is correct, then our results suggest that GRIP domain proteins may function either in recycling of proteins from endosomes to the TGN, in maintaining TGN structure, or both.

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MASTER PAGES

Characterization of Mouse tGolgin-1 (Golgin-245/*trans*-Golgi p230/256 kD Golgin) and Its Upregulation during Oligodendrocyte Development

DAVID A. COWAN, DENISE GAY, BERT M. BIELER, HUIZHEN ZHAO, ATSUKO YOSHINO, JAMES G. DAVIS, MARY M. TOMAYKO, RAMACHANDRAN MURALI, MARK I. GREENE, and MICHAEL S. MARKS

ABSTRACT

As part of an effort to identify gene products that are differentially regulated during oligodendrocyte development, we isolated a mouse cDNA that encodes tGolgin-1, a homolog of the human protein known as golgin-245, *trans*-golgi p230, or 256 kD golgin. Human tGolgin-1 is the target of autoantibodies in patients with Sjögren's syndrome, and is thought to be involved in vesicular transport processes at the *trans*-Golgi network. Sequencing of cDNAs and EST clones comprising the full-length tGolgin-1 transcript predict marked homology with the amino- and carboxy-terminal regions of the human protein, but more limited homology within the central predicted coiled-coil region. Epitope tagged, truncated forms of mouse tGolgin-1, like those of its human homolog, were localized at steady state to the Golgi/*trans*-Golgi network in transfected cells. The tGolgin-1 message was expressed in all tissues examined, but was highly upregulated in oligodendrocyte precursors at a stage just prior to myelination. This expression pattern suggests that tGolgin-1 may play a role in specialized transport processes associated with maturation and/or differentiation of oligodendrocyte precursors.

INTRODUCTION

Oligodendrocytes are responsible for the highly complex process of myelination within the central nervous system. Oligodendrocytes arise from undifferentiated neuroepithelial precursors within the ventricular zone of the spinal cord (Orentas and Miller, 1998). These precursors undergo a complex developmental program during which they differentiate first into highly proliferative, pluripotent migratory cells, and then into nonmotile, immature oligodendrocytes that make direct physical contacts with target neurons (Pfeiffer *et al.*, 1993; McMorris and McKinnon, 1996). Mature oligodendrocytes synthesize myelin components, such as myelin basic protein, myelin-associated glycoprotein, myelin/oligodendrocyte protein, and proteolipid protein, which are incorporated into myelin-rich regions of the plasma membrane that enwrap axons of adjacent neurons (Campagnoni, 1988). These developmental changes are marked by the expression of distinct cell surface markers, particularly glycolipids or glycoproteins, and are accompanied by dramatic changes in cellular morphology and physiology. These changes include the extension of cell processes, changes in expression of adhesion molecules and growth factor receptors, and ultimately, the generation and compaction of myelin sheets at great distances from the cell body (Pfeiffer *et al.*, 1993).

The developmental changes in the physiologic and morphologic characteristics of oligodendrocyte precursors likely reflect alterations in the cytoskeleton and in the biosynthetic machinery of the cell. Indeed, unusual microtubule arrays (Lunn *et al.*, 1997) and peripheral Golgi-like structures (de Vries *et al.*, 1993) have been found in the extended processes of oligodendrocyte precursors. Furthermore, oligodendrocytes at later stages of development display a number of features characteristic of polarized cells (Pfeiffer *et al.*, 1993), such as differential sorting of viral glycoproteins (de Vries *et al.*, 1998). The generation of these unusual morphologic features and protein sorting pathways must be orchestrated within the cytoplasm and the central vacuolar system by structural and regulatory proteins, the expression of which is also likely to be developmentally regulated. For example, Rab3a and Rab3c, proteins respectively as-

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sociated with synaptic and endocrine secretory vesicles, are upregulated during later oligodendrocyte development (Madison *et al.,* 1996), and a specialized Rab protein, Rab22b, is expressed selectively in oligodendrocyte lineage cells (Rodriguez-Gabin *et al.,* 2001). It would be expected that Rab effector proteins and other components of the trafficking machinery would also be differentially regulated in these cells.

We previously described a novel cell surface determinant, termed OlP-1 (Oligodendrocyte Precursor protein-1), that is specifically expressed on developing oligodendrocyte precursors at a stage just prior to the onset of synthesis of myelin components (Gay *et al.,* 1997). In an effort to characterize the OlP-1 antigen, we used an anti-OlP-1 monoclonal antibody (mab) to screen a cDNA expression library from a postnatal day 8 mouse brain. Using this approach, we identified a set of cDNA clones that encode partial, alternatively spliced forms of a single gene product. Sequence analysis of cDNAs spanning the entire coding region identified the gene product as the murine homolog of a human protein previously identified as golgin-245 (Fritzler *et al.,* 1995), *trans*-golgi p230 (Kooy *et al.,* 1992), or 256 kD golgin (H. P. Seelig, Genbank accession #X82834) and referred to here as tGolgin-1. tGolgin-1 is a peripheral membrane protein of unknown function found on the cytosolic face of the *trans*-Golgi network (TGN) and associated vesicles (Gleeson *et al.,* 1996). It is part of a family of peripheral membrane proteins containing a large, central coiled-coil region and a C-terminal "GRIP" domain that is responsible for localization to the Golgi and/or TGN (Barr, 1999; Kjer-Nielsen *et al.,* 1999a, 1999b; Munro and Nichols, 1999; Brown *et al.,* 2001). tGolgin-1 mRNA was ubiquitously expressed, but was dramatically upregulated in oligodendrocyte precursors at the stage of development marked by expression of the OlP-1 determinant. We postulate that upregulation of tGolgin-1 during this stage of development facilitates either the establishment of peripheral Golgi-like structures in oligodendrocyte processes or the synthesis, targeting, or recycling of myelin constituents in preparation for myelin formation.

MATERIALS AND METHODS

AU

Animals and tissue culture conditions

Virus-free BALB/c postnatal day 1 mice (Harlan, Indianapolis, IN) provided cerebral tissue for all primary oligodendrocyte and astrocyte cultures. Oligodendrocyte precursor culture conditions were as described in Gay *et al.* (1997). Cerebral cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) for 2–4 weeks to generate enriched astrocyte populations. The BALB/C7 fibroblast and HeLa cell lines (ATCC) were maintained in DMEM/10% FBS.

Indianapolis, IN

cDNA library production, screening, and sequencing

mRNA was extracted from brain tissue of postnatal day (P)8 mice using the Fastrack system (Invitrogen, Carlsbad, CA), converted into cDNA and directionally cloned using the ZAP Express cDNA II cloning kit (Stratagene, La Jolla, CA). Recombinant fusion proteins were screened for binding to anti-OlP-1 mab with the Picoblue Immunoscreening Kit (Stratagene). These original cDNAs were sequenced using automated sequencing facilities and T3, T7, and internal designed primers. Sequences were analyzed using Geneworks and BLAST software. Additional regions were characterized following identification of mouse EST clones with homology to regions of the human p230 cDNA further 5′ than the original mouse cDNA clones. EST clones with accession numbers (Acc) AA144704, AA561361, and AA389589 were obtained from Genome Systems Inc. (St. Louis, MO). The region between EST clone AA144704 and cDNA clone C91 was amplified by reverse transcriptase (RT) coupled polymerase chain reaction (PCR) from neonatal mouse brain RNA as described (Marks *et al.,* 1995) and cloned using unique NheI and PshAI restriction sites. The region encompassing EST clone AA098411 up to the NdeI site of EST clone AA144704 was also generated by RT-PCR from neonatal mouse brain RNA. The region corresponding to the coding region for the N-terminus of human p230 was generated by RT-PCR from adult mouse brain and liver mRNA using a degenerate oligonucleotide encoding the N-terminal human p230 residues and a reverse primer downstream of the NdeI site. The sequence of the 5′ UTR and the translation start site were inferred from overlapping genbank database entries (Acc BC003268, BF162586, BI151932, and BE304004). See Figure 1 for schematic diagram. Oligonucleotide sequences are available upon request. Sequences of amplified regions were confirmed from at least three cloned products of at least two separate RT-PCR reactions, and only sequences confirmed by two or more database entries are listed for the 5′ UTR. Coiled-coil predictions were done using the MTIDK matrix within the COILS v2.2 program from the Swiss Institute for Experimental Cancer Research (http://www.isrec.isb-sib.ch/software/COILS_form.html). Fig. 1

Northern analysis

RNA was isolated from the indicated tissues of female Balb/c mice using the FastTrack 2.0 kit (InVitroGen) for poly(A)+ RNA or the RNA STAT-60 kit (Tel-Test, Inc., Friendswood, TX) for total RNA. RNA samples (25 μg total or 2.5 μg polyA+) were fractionated on 0.8–0.9% agarose gels in formaldehyde/MOPS essentially as described (Sambrook *et al.,* 1989). Gels were soaked in 0.05 M sodium hydroxide for 20 min, then 20× SSC for 1 h prior to transfer onto Immobilon Ny+ filters (Millipore, Bedford, MA) overnight in 20× SSC. Blots were washed in DEPC-treated H_2O, and then baked at 80°C for 2 h. Digoxigenin-labeled probes were generated by PCR using the PCR DIG Probe Synthesis Kit from Boehringer Mannheim-Roche (Palo Alto, CA), and hybridized to filters and developed using the DIG Luminescent Detection Kit according to the manufacturer's instructions. Bands were visualized using a Molecular Dynamics (Sunnyvale, CA) Storm 760 Phosphor Imager and ImageQuant v. 1.1 software. The probe for tGolgin-1 encompassed nt. 110–1172, and the probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) encompassed the entire open reading frame (nt. 47 to 1045).

HA-tagged constructs

HA11 epitope tag sequences were constructed by PCR for ligation onto the N- or C-terminal coding regions of the clone C91 cDNA sequence. For an N-terminal tag, a PCR product including a 5′ Kozak consensus sequence, the coding region for an HA11 tag, and a region contiguous with the 5′ sequence for C91 was subcloned into the SalI and SphI sites of C91 to gen-

erate HA-tGolgin-C1247. For a C-terminal tag, a PCR product contiguous with the coding region for the C-terminus of C91 and containing a 3′ HA tag followed by a stop codon was cloned into the Nru1 and Xba1 sites of C91 to generate tGolgin-C1247-HA. Resultant constructs were subcloned into the pCDM8.1 expression vector (Bonifacino *et al.*, 1990). Additional truncation products were generated by PCR from the original clones using similar designed primers, and all amplified products were verified by automated DNA sequencing.

Immunofluorescence microscopy

HeLa cells plated on glass coverslips in six-well dishes were transiently transfected using the calcium phosphate method (Sambrook *et al.*, 1989), fixed with 2% formaldehyde 40 h post-transfection, and processed for indirect intracellular immunofluorescence microscopy as previously described (Marks *et al.*, 1995). Antibodies included a rabbit antiserum to TGN46 (Prescott *et al.*, 1997) (a gift of V. Ponnambalam, Univ. Dundee), a rabbit antiserum to the β-COP subunit of COPI (a gift of Dr. J. Lippincott-Schwartz, National Inst. of Health, Bethesda, MD), anti-HA11 monoclonal antibody (BabCo, Richmond, CA) and secondary fluorochrome-coupled antibodies (Jackson Immunoresearch, West Grove, PA). Slides were analyzed on a Zeiss Axioplan fluorescence microscope and photographic images were digitized using a Nikon LS-1500 film scanner. In one experiment, images were captured with a Leica TCS laser confocal scanning microscope equipped with a 100×, 1.4 N.A. oil immersion lens and the Leica TCS software package.

ES, OK

(Zeiss, Thornwood, NY)

Au: OK?

Flow cytometry and cell sorting

Precursors from day 4 (populations 1 and 2) or day 9 (population 3) mixed mouse oligodendrocyte cultures were incubated with combinations of anti-OIP-1 (Gay *et al.*, 1997), anti-O4, R-Mab (Ranscht *et al.*, 1982) and O1 (anti-GalC) mabs and prepared for cytofluorography as described (Gay *et al.*, 1997). Cell sorting was accomplished using a FACStar Plus (Becton Dickenson, Franklin Lakes, NJ) and CellQuest software.

mouse

Semiquantitative RT-PCR

mRNA was extracted from 10^5 cells using Micro-FastTrack (Invitrogen), converted to cDNA with Superscript II reverse transcriptase (Life Technologies, Rockville, MD) and PCR-amplified using the High Fidelity PCR system (Roche Applied Science, Indianapolis, IN). Semiquantitative PCR was carried out on 5×10^3 cell equivalents with an α^{32}P dCTP tracer using previously specified conditions (Tomayko and Cancro, 1998). Samples (2 μl) were removed at designated cycles, electrophoresed onto a 0.8% agarose gel, vacuum dried and subjected to autoradiography. Nonquantitative PCR was carried out for 40 cycles using mRNA from approximately 10^5 cells. The cDNA 1 PCR product was TA cloned and sequenced to show 100% identity to cDNA clone C91 (unpublished data). The actin primers were as specified (Tomayko and Cancro, 1998). The PLP primers were 5′gactacaagaccaccatctg3′ (nt. 309 to 328 and 5′gaagagggccaatcagt ggca3′ (nt. 923 to 943). The cDNA 1 primers for tGolgin-1 were 5′ggctgggagagctgaggcaga3′ (nt. 3260 to 3280) and 5′gttcaagctcttgtcctccag3′ (nt. 3640 to 3660). The cDNA 2 primers were 5′tggttcagagacttcagcact3′ (nt. 4730 to 4750) and 5′tgtgcagtcctgggaatctg3′ (nt. 5216 to 5235).

RESULTS

Isolation of a tGolgin-1 cDNA by screening a postnatal mouse brain cDNA library with anti-OIP-1

OIP-1 was identified as a developmentally regulated cell surface determinant specific to premyelinating oligodendrocyte precursors (Gay *et al.*, 1997). Because these cells are most highly represented in the mouse brain during early postnatal development, a postnatal day 8 mouse brain cDNA expression library was screened with anti-OIP-1 mab. Three independent cDNAs (clones C95, C49, and C91) were retrieved ranging in size from 2.2 kb to over 4 kb. Sequencing showed them to be identical except for a 63 bp deletion at the 3′ end of the largest cDNA clone (C91). Figure 1a shows the relative sizes and sequence overlap of these cDNAs. The sequence of C91 contained a single open reading frame of 3741 nucleotides extending to the extreme 5′ end. The 3′ terminus was verified by the presence of multiple stop codons and a poly A tail.

Comparison of the predicted translation product of the C91 open reading frame to available databases identified three entries with considerable homology: golgin 245 (accession number U31906; Fritzler *et al.*, 1995), 256 kD golgin (acc. no. X82834), and *trans*-golgi p230 (acc. no. U41740) (Erlich *et al.*, 1996), corresponding to products of the *GOLGA*4 gene. These three molecules bear identical nucleotide sequences except for deletions within the 3′ and/or 5′ regions, and we, therefore, believe that they represent the product of a single gene with a single alternative splice site near the 3′ end (see Discussion). Because of their immunolocalization to the TGN (Gleeson *et al.*, 1996), we refer to them collectively as tGolgin-1. The region of homology extended through the entire predicted translation product of C91, but corresponded to only the C-terminal 1247 amino acids of the 2228–2230 residue full-length tGolgin-1 protein. This suggested that C91 was not a full-length cDNA for mouse tGolgin-1.

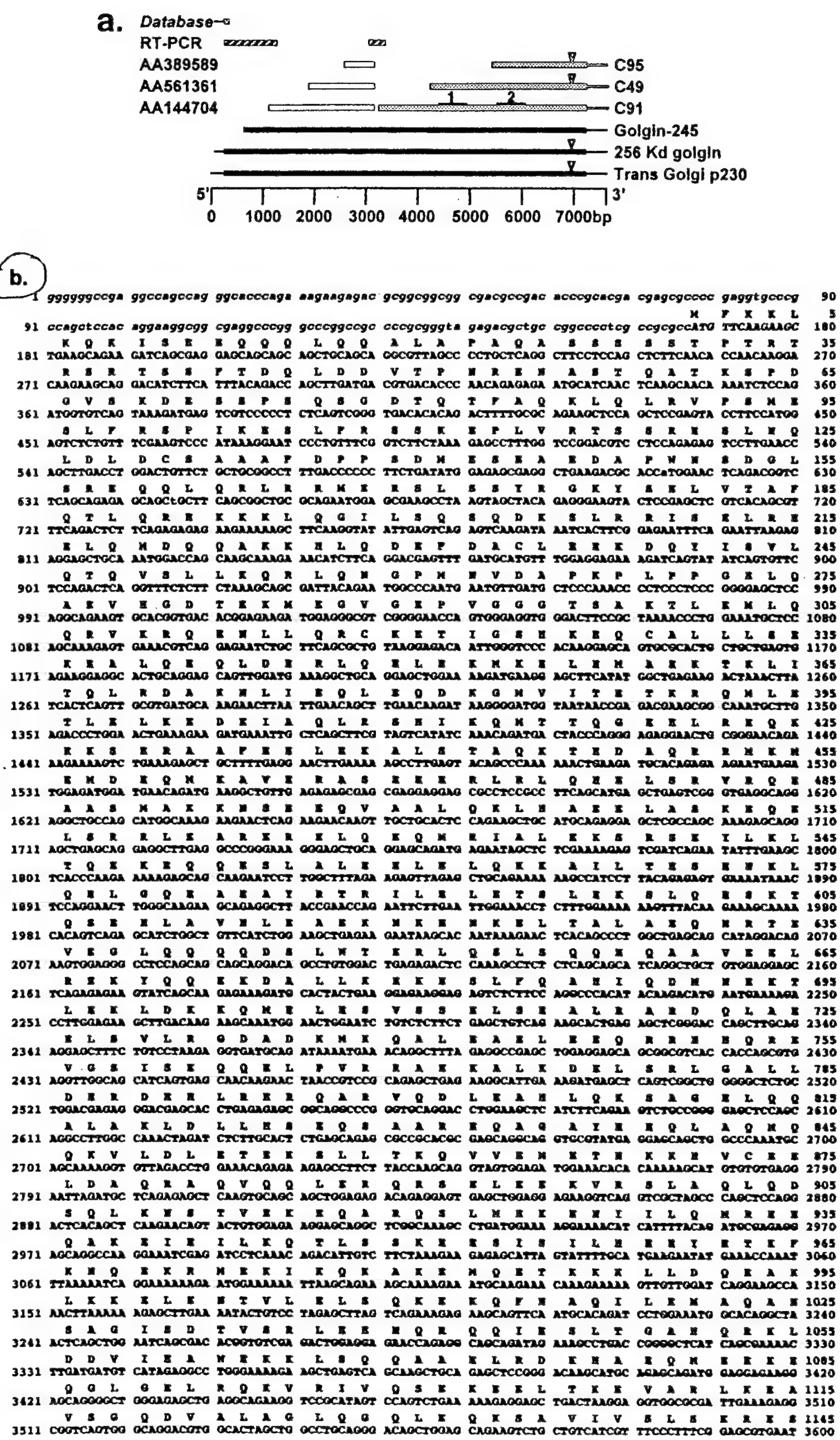
A BLAST search of the mouse EST database revealed numerous additional clones with predicted translated amino acid sequence similarity to regions within the N-terminal 1000 residues of human tGolgin-1. Two of these clones, Acc AA098411 and AA144704, were retrieved, and together spanned sequences encoding 878 additional contiguous residues N-terminal to those encoded by C91 (Fig. 1a). cDNA sequences between EST clone AA144704 and C91 were amplified by RT-PCR from postnatal mouse brain RNA, and EST clone AA098411 was reconstructed using RT-PCR from the same source using the available sequence deposited in Genbank. The coding region for the predicted N-terminal region was amplified by RT-PCR of prenatal mouse brain RNA using a degenerate primer corresponding to the coding region for the first six amino acids of human tGolgin-1 and a second reverse primer from within the coding region of EST clone AA144704. The remaining 5′ UTR and coding region for the first six amino acids are inferred from overlapping entries in the Genbank database (see Materials and Methods for details). RT-PCR of mRNA from several sources indicated that all separately identified regions were colinear in a single RNA species (see below). We refer to this composite clone as mouse tGolgin-1.

The complete cDNA sequence and deduced amino acid sequence of mouse tGolgin-1 is listed in Figure 1b, and the deduced amino acid sequence is compared with that of human tGolgin-1 in Figure 2A. The mouse tGolgin-1 open reading

Fig 2

Comp's Shading on J-print is too light

enlarge to match "a"



QU2

OK

FIG. 1.

```
           Q  L  Q   S  Q  V   E  K  L  E   A  D  L   G  C  S   L  S  E  K   L  S  L   Q  E  E   L  A  E  L 1175
3601 CTCAGCTGCA GTCACAAGTG GAAAAGCTGG AAGCTGATTT GGGTTGTTCT CTGAGTGAAA AGCTTTCCCT TCAAGAAGAG CTGGCTGAGC 3690
           K  L  L   A  D  K   S  Q  L  R   V  S  E   L  T  G   Q  V  Q  A   A  E  K   E  L  Q   S  C  K  S 1205
3691 TGAAACTGCT GGCAGACAAG AGTCAGCTGA GGGTCTCCGA GCTGACGGGC CAGGTGCAGG CTGCGGAGAA GGAGCTCCAG AGCTGTAAGT 3780
           L  H  E   L  S  K   K  S  L  E   D  K  S   L  N  L   K  S  L  L   E  E  L   A  S  Q   L  D  S  R 1235
3781 CTCTGCATGA GCTCAGCAAG AAGAGCCTGG AGGACAAGAG CTTGAACCTC AAAAGCTTGC TTGAGGAGCT AGCATCTCAG CTAGACAGTC 3870
           C  E  R   T  K  A   L  L  E  A   K  T  N   E  L  V   C  T  S  R   D  K  A   D  A  I   L  A  R  L 1265
3871 GCTGTGAGAG AACCAAAGCT CTGTTAGAAG CCAAGACGAA CGAACTAGTG TGCACCAGCC GTGACAAAGC CGACGCTATT CTCGCTAGGC 3960
           S  Q  C   Q  R  H   T  A  T  V   G  E  A   L  L  R   R  M  G  Q   V  S  E   L  E  A   Q  L  T  Q 1295
3961 TGTCCCAATG CCAGCGCCAC ACAGCCACAG TTGGGGAGGC GCTGCTCAGG AGAATGGGCC AGGTTTCTGA GTTAGAAGCA CAACTTACAC 4050
           L  T  E   E  Q  R   T  L  K  S   S  F  Q   Q  V  T   N  Q  L  E   E  K  E   K  Q  I   K  T  M  K 1325
4051 AGCTGACAGA GGAGCAGCGC ACACTAAAGA GCTCTTTTCA GCAGGTTACC AATCAGCTGG AAGAGAAAGA GAAGCAGATT AAGACCATGA 4140
           A  D  I   E  G  L   L  T  E  K   E  A  L   Q  Q  E   G  G  Q  Q   R  Q  A   A  S  E   K  E  S  C 1355
4141 AGGCTGACAT TGAAGGTCTT CTCACAGAAA AAGAAGCCTT ACAGCAAGAA GGAGGCCAGC AGCGGCAGGC GGCCTCTGAG AAGGAGTCCT 4230
           I  T  Q   L  K  K   E  L  A  E   N  I  N   A  V  T   L  L  R  E   E  L  S   E  K  K   S  E  I  A 1385
4231 GCATCACACA GCTGAAGAAA GAGTTAGCGG AGAACATCAA TGCCGTCACC CTACTGAGAG AAGAGCTGTC GGAGAAGAAG TCTGAGATTG 4320
           S  L  S   K  Q  L   S  D  L  G   A  Q  L   E  S  S   I  S  P  S   D  K  A   E  A  I   S  A  L  S 1415
4321 CCAGTCTTAG CAAGCAGTTA AGTGATCTCG GTGCTCAGCT GGAGAGCAGC ATCAGCCCGA GTGACAAGGC CGAAGCCATC TCTGCCCTGA 4410
           K  Q  H   E  E  Q   E  L  Q  L   Q  A  Q   L  Q  E   L  S  L  K   V  D  A   L  S  K   E  K  M  S 1445
4411 GCAAGCAGCA CGAGGAACAA GAGCTGCAGC TGCAAGCTCA GCTTCAGGAG CTGTCTCTGA AAGTCGATGC TCTGAGTAAA GAGAAAATGT 4500
           A  L  E   Q  V  D   H  W  S  N   K  F  S   E  W  K   K  K  A  Q   S  R  L   A  Q  H   Q  S  T  I 1475
4501 CTGCCCTTGA GCAGGTGGAT CATTGGTCCA ACAAGTTCTC AGAGTGGAAG AAAAAAGCAC AGTCCAGACT TGCCCAGCAC CAAAGCACTA 4590
           K  D  L   Q  A  Q   L  D  V  K   A  T  D   A  R  E   K  E  E  Q   I  C  L   L  K  E   D  L  D  R 1505
4591 TTAAAGACTT GCAGGCACAG CTAGACGTAA AAGCCACGGA CGCTCGTGAG AAGGAGGAGC AGATATGTTT ACTGAAGGAA GACCTTGATC 4680
           Q  N  K   K  F  E   C  L  K  G   E  M  E   V  R  K   S  K  M  E   K  K  E   C  D  L   E  T  A  L 1535
4681 GGCAGAATAA AAAGTTTGAA TGTTTAAAAG GCGAGATGGA AGTCAGGAAG AGCAAGATGG AGAAAAAGGA GTGTGACTTA GAGACTGCGT 4770
           K  T  Q   T  A  R   V  V  E  L   E  D  C   V  T  Q   R  K  K  E   V  E  S   L  N  E   T  L  K  N 1565
4771 TAAAGACTCA GACAGCAAGG GTCGTTGAAT TAGAAGACTG CGTTACTCAG AGGAAAAAAG AAGTTGAGTC CCTAAATGAA ACACTTAAGA 4860
           Y  N  Q   Q  R  D   T  E  H  S   G  L  V   Q  R  L   Q  H  L  E   E  L  G   E  E  K   D  N  K  V 1595
4861 ATTACAACCA GCAGAGGGAC ACTGAGCACA GTGGGCTGGT TCAGAGACTT CAGCACTTGG AAGAGTTGGG AGAAGAGAAA GACAACAAGG 4950
           R  E  A   E  E  T   V  L  R  L   R  E  H   V  S  S   L  E  A  E   L  G  T   V  K  K   E  L  E  H 1625
4951 TTAGGGAGGC TGAAGAGACC GTCCTGAGAC TACGAGAGCA CGTGTCCTCG CTGGAAGCTG AACTTGGAAC TGTGAAGAAG GAACTAGAAC 5040
           V  N  S   S  V  K   S  R  D  G   E  L  K   A  L  E   D  K  L  E   L  E  S   A  A  K   V  E  L  K 1655
5041 ATGTAAATTC AAGTGTGAAA AGCAGAGATG GGGAGCTGAA AGCTTTAGAA GACAAACTTG AGTTAGAAAG TGCTGCAAAA GTGGAGCTGA 5130
           R  K  A   E  Q  K   I  A  A  I   R  K  Q   L  L  S   Q  M  E  E   K  T  Q   R  Y  A   K  D  T  E 1685
5131 AGAGGAAGGC CGAGCAGAAG ATCGCTGCCA TCAGGAAGCA GCTCTTGTCT CAGATGGAAG AGAAAACCCA GCGGTATGCG AAGGACACAG 5220
           N  R  L   S  E  L   S  A  Q  L   K  E  R   E  K  Q   V  H  S  L   E  D  K   L  K  N   L  E  S  S 1715
5221 AGAACCGACT GAGTGAGCTG AGTGCACAAT TAAAAGAAAG AGAAAAGCAA GTTCACAGCC TAGAAGACAA ACTTAAAAAC CTGGAGAGTT 5310
           P  H  P   E  V  P   A  V  S  R   S  M  Q   S  V  A   A  S  P  E   Q  E  A   P  D  S   Q  D  C  T 1745
5311 CTCCACACCC AGAAGTGCCA GCGGTGTCCA GATCTATGCA AAGTGTGGCA GCGTCTCCCG AGCAAGAGGC TCCAGATTCC CAGGACTGCA 5400
           H  K  A   C  K  E   R  L  C  M   L  Q  R   R  L  S   E  K  E  K   L  L  R   R  L  E   Q  G  E  G 1775
5401 CACACAAGGC CTGTAAAGAA AGACTCTGCA TGCTGCAAAG ACGTTTAAGT GAAAAAGAGA AGCTGCTGCG CAGGCTGGAG CAGGGCGAAG 5490
           E  A  R   P  S  Q   P  E  A  Q   H  R  A   L  S  G   K  L  D  C   T  R  A   R  Q  L   E  D  H  V 1805
5491 GCGAGGCGAG GCCATCGCAG CCTGAGGCTC AGCACAGGGC GCTCTCTGGA AAGTTAGACT GCACTAGAGC CAGGCAGCTT GAAGATCACG 5580
           L  I  G   C  L  P   E  E  L  E   E  K  M   K  C  S   L  I  V  S   Q  P  M   G  E  E   T  G  N  N 1835
5581 TTTTGATAGG ATGTCTTCCA GAAGAACTCG AAGAAAAGAT GAAATGTTCC TTAATTGTGT CTCAGCCCAT GGGAGAAGAA ACTGGTAACA 5670
           T  G  V   K  Q  N   W  A  S  V   V  D  S   V  Q  K   T  L  Q  E   K  E  L   T  C  Q   A  L  E  Q 1865
5671 ACACAGGGGT GAAGCAGAAT TGGGCAAGTG TGGTTGACAG TGTTCAGAAA ACCCTCCAGG AAAAGGAGCT CACTTGCCAG GCCCTAGAGC 5760
           R  V  K   E  L  E   S  D  L  V   R  E  R   G  A  H   R  L  E  V   E  K  L   T  L  K   Y  E  K  S 1895
5761 AAAGGGTGAA AGAACTGGAG TCGGACTTAG TAAGAGAGAG GGGCGCCCAT AGACTTGAAG TGGAAAAGTT GACCTTAAAA TATGAAAAAT 5850
           Q  S  S   Q  Q  E   M  D  G  E   N  K  C   V  E  V   L  E  D  R   P  E  E   N  S  Q   S  H  E  I 1925
5851 CGCAATCTTC CCAGCAAGAA ATGGATGGGG AAAATAAATG TGTAGAAGTG TTGGAAGACA GGCCTGAGGA AAACTCCCAA TCCCATGAGA 5940
           Q  S  N   V  G  T   V  D  G  L   R  S  D   L  E  S   K  L  T  G   A  E  R   D  K  Q   K  L  S  K 1955
5941 TCCAGTCAAA CGTGGGGACC GTGGACGGCC TGCGCAGCGA CCTGGAGTCC AAGTTGACAG GAGCGGAGCG GGACAAGCAG AAGCTGAGCA 6030
           E  V  A   R  L  Q   K  E  L  R   A  L  R   R  E  H   Q  Q  E  L   D  I  L   K  R  E   C  E  Q  E 1985
6031 AGGAGGTGGC GAGGCTGCAG AAGGAGCTGC GAGCTCTGCG GAGGGAGCAC CAGCAGGAGC TGGACATCCT GAAGAGGGAG TGCGAGCAGG 6120
           A  E  E   K  L  K   Q  E  Q  E   D  L  E   L  K  H   T  S  T  L   K  Q  L   M  R  E   F  N  T  Q 2015
6121 AGGCCGAGGA AAAGCTCAAA CAGGAGCAAG AGGATCTTGA GTTGAAGCAC ACGTCCACAC TGAAGCAGCT GATGCGGGAG TTTAACACAC 6210
           L  A  Q   K  E  Q   E  L  E  R   T  V  Q   E  T  I   D  K  A  Q   E  V  E   A  E  L   L  E  S  H 2045
6211 AACTGGCACA GAAGGAGCAG GAGCTGGAAA GAACTGTCCA GGAGACCATT GATAAGGCCC AAGAGGTGGA AGCCGAACTT CTGGAAAGCC 6300
           Q  E  E   T  Q  Q   L  H  R  K   I  A  E   K  E  D   D  L  R  R   T  A  R   R  Y  E   E  I  L  D 2075
6301 ACCAAGAAGA GACACAGCAG TTGCATAGAA AGATCGCGGA GAAAGAGGAT GATCTGAGAA GGACAGCCAG GAGATACGAG GAGATACTCG 6390
           A  R  E   E  E  M   T  G  K  V   T  D  L   Q  T  Q   L  E  E  L   Q  K  K   Y  Q  Q   R  L  E  Q 2105
6391 ATGCCCGTGA AGAAGAAATG ACTGGCAAAG TGACGGACCT GCAGACCCAG CTCGAGGAGC TCCAGAAGAA ATACCAGCAG AGGCTGGAGC 6480
           E  E  S   T  K  D   S  V  T  I   L  E  L   Q  T  Q   L  A  Q  K   T  T  L   I  S  D   S  K  L  K 2135
6481 AGGAGGAGAG CACCAAGGAC AGTGTAACAA TTTTGGAGCT ACAGACACAA CTAGCGCAGA AGACCACTCT GATCAGCGAC TCCAAGCTGA 6570
           E  Q  E   L  R  E   Q  V  H  N   L  E  D   R  L  K   R  Y  E  K   N  A  C   A  A  T   V  G  T  P 2165
6571 AGGAGCAGGA GTTGAGAGAA CAGGTCCATA ATTTAGAAGA CCGTTTGAAA AGATATGAAA AGAATGCATG TGCAGCAACT GTGGGGACAC 6660
           Y  K  G   G  N  L   Y  H  T  E   V  S  L   F  G  E   P  T  E  P   E  Y  L   R  K  V   M  F  E  Y 2195
6661 CTTACAAAGG TGGCAATTTG TACCACACTG AGGTCTCACT CTTCGGAGAA CCTACCGAGT TTGAGTATTT GCGAAAAGTG ATGTTTGAAT 6750
           M  M  G   R  E  T   K  T  M  A   K  V  I   T  T  V   L  K  F  P   D  D  Q   A  Q  K   I  L  E  R 2225
6751 ATATGATGGG TCGCGAGACT AAGACCATGG CCAAAGTTAT AACCACTGTC CTGAAATTCC CTGATGATCA GGCTCAGAAA ATTTTGGAAA 6840
           E  D  A   R  L  M   S  W  L  R   T  S  S   *                                                   2238
6841 GAGAAGATGC TCGTCTGATG TCATGGCTCC GAACCTCATC TTGAagaaat ggagagagtg agctaggcag ccgctgcctg ggaagctgtc 6930
6931 cactgtgggc ttttagagaa tggcgctgtc attacagcca ttggtgtagc ttgaaggcag atgctactct ggcctcagat acattgctgt 7020
7021 ggaagcaggc gcttttggtg gggagatggt ggtgggtttg tcctttttta ttcagttttt tcttgggaag ttttctgtgg tttaaaggct 7110
7111 gttctgactg gccccacctg gcttggctct tcagtgtctt tttcttgtgt tctccctctc tttaaagaac tgtccatctt ccttatttat 7200
7201 tttagggtgg tttctttttc ttcctaaaga cttgtgcaat atattttgag gtgaaactta gtgaattttt ttctgataaa ttagagattt 7290
7291 taattgactg tttcacagat tgattttttg aatatttgct aaagcctagt tctttatgct atgataagtc ccaaatggca gtacctcatg 7380
7381 tttacctagc ttttggactt atattatttt tcagaggaaa aaatactact gtaaattgta aatagtacat aatcctattg tctgcagctc 7470
7471 tgtgactgtt ggccatgtca tctcagagga tcagataaat aaagtttatt tcctataaaa                                  7530
```

99 1 2

FIG. 1. (*Continued*) Isolation and sequence of mouse tGolgin-1 cDNA. (a) Schematic representation of regions of sequence overlap between *trans*-golgi p230, golgin-245, 256 kD golgin, isolated OIP-1 cDNA clones (C91, C49, and C95), EST clones (AA144704, AA561361, and AA389589), and additional regions identified in this work. The triangle represents the 63 bp insertion in p230 and clones C95 and C49. ~~(a)~~ 1 and ~~(b)~~ 2 represent the locations of PCR products used in Figure 7. Regions identified by RT-PCR only are indicated. *Database* represents the 5′ UTR and translation start sequences that were deduced from the deposited sequences of Genbank entries BC003268, BF162586, BI151932, and BE304004; these sequences were not confirmed by our direct sequencing, but represent overlap of the corresponding database entries. (b) Nucleotide sequence of mouse tGolgin-1 cDNA and deduced amino acid sequence. Italics represent the inferred sequences from the database entries as indicated above.

frame encodes 2238 amino acids with a predicted molecular mass of 258 kDa, compared to 2230 amino acids and 261 kDa for the human homolog. The predicted amino acid sequences of the two homologs show 70% identity and 85% similarity. Interestingly, *trans*-golgi p230 and 256 kD golgin, like mouse tGolgin-1 cDNA cones C49 and C95, maintained the 65 bp 3' nucleotide stretch encoding "FTSPRSGIF" at the C-terminus, whereas golgin-245 and clone C91 had this region deleted, generating an alternative "SWLRTSS" C-terminal end. Both human and mouse proteins are predicted to consist primarily of coiled-coil domains, with short regions of potentially globular structure at the N- and C-termini (Fig. 2B).

To determine whether the sequences we identified corresponded to the only mouse homolog of human tGolgin-1, we searched the dbest database for homologous murine EST sequences using BLAST. We retrieved 145 nucleotide sequences that were greater than 93% identical to mouse tGolgin-1, most of which were mismatched at only one to three residues; given the distribution of the nucleotide differences, we suspect that these clones contain identical sequences to those in mouse tGolgin-1 and that differences reflect sequencing errors and/or potential haplotype variations. Included among the retrieved sequences were clones homologous to regions spanning the entire mouse tGolgin-1 cDNA, with the exception of the short adenosine-rich stretch between C91 and AA144704. Searches of the dbest database for translated sequences homologous to either the human tGolgin-1 proteins or the translated mouse tGolgin-1 sequence resulted in no additional entries other than those identified on the basis of nucleotide similarity. This suggests that no other tGolgin-1 homologues have yet been entered in the EST database. The fact that they were obtained from highly diverse cDNA library sources suggests that they represent an ubiquitous mRNA, as would be expected for a tGolgin-1 homolog (see below).

Significant but low homology was also observed between the mouse tGolgin-1 deduced amino aid sequence and the C-terminal half of myosin II (unpublished data). This homology, which ranged from 23–45% over four distinct regions, correlated specifically with predicted coiled-coil regions as assessed using the MTIDK matrix (Lupas *et al.*, 1991) (Fig. 2B). The GCG computer program "Moment" used to calculate helical dipole moments to predict α-helical regions failed to find long helices within the tGolgin-1 sequence (unpublished data). These data cannot distinguish between the possibilities that tGolgin-1 is supercoiled like the myosin heavy chain C-terminal domain or globular with many small coiled-coil–like domains. However, because myosin II and several other coiled-coil containing Golgi- and endosome-associated peripheral membrane proteins are believed to form dimeric supercoiled structures, we anticipate that tGolgin-1 has a similar structure.

tGolgin-1 localization to the TGN through the C-terminal half of the molecule

Because human tGolgin-1 has been shown to be peripherally associated with the TGN (Gleeson *et al.*, 1996), we assayed for the intracellular localization of the mouse tGolgin-1 C-terminal fragment encoded by C91. Expression vectors encoding HA11 epitope-tagged forms of the translation product of C91 (HA-C1247, representing the C-terminal 1247 aa of tGolgin-1) were transiently transfected into HeLa cells, and localization was determined by indirect intracellular immunofluorescence microscopy (IFM) using anti-HA11 antibodies. As shown in Figure 3, HA-C1247 localized to a paranuclear, reticular structure reminiscent of the Golgi complex. Parallel staining with an antiserum to the endogenous TGN resident protein, TGN46 (Fig. 3A and B), or to the Golgi stack-associated β-COP subunit of coatomer (Duden *et al.*, 1991) (Fig. 3C and D) showed extensive overlap, suggesting that the C-terminal tGolgin-1 fragment primarily localizes to the TGN and/or the Golgi stacks. Similar results were obtained using C1247 tagged at the C-terminus with the HA11 epitope (unpublished data). Like human tGolgin-1 (Gleeson *et al.*, 1996), the TGN-associated HA-C1247 was partially dispersed upon treatment with brefeldin A (unpublished data). These data further support the notion that we identified the mouse homolog of human tGolgin-1, and that tGolgin-1 localizes to the TGN.

Fig. 3

The C-terminal 80 amino acids of human tGolgin-1 encompass a GRIP domain, a modestly conserved region that is necessary and sufficient to mediate localization to the TGN (Barr, 1999; Kjer-Nielsen *et al.*, 1999a, 1999b; Munro and Nichols, 1999). To determine whether the comparable region of mouse tGolgin-1 was sufficient to mediate Golgi localization, we generated N-terminally HA-tagged truncation mutants containing the C-terminal 312, 186, or 81 amino acids of mouse tGolgin-1, and expressed them by transient transfection in HeLa cells. As shown in Figure 4a–f, immunofluorescence microscopy analyses indicated that all of these constructs colocalized well with TGN46. Mutagenesis of a critical conserved tyrosine residue at position 2187 completely disrupted localization, resulting in a diffuse cytoplasmic staining pattern (Figure 4g and h). These results indicate that mouse tGolgin-1 retains a functional GRIP domain, and that the C-terminal 81 amino acids are sufficient to specify localization to the Golgi/TGN.

Fig. 4

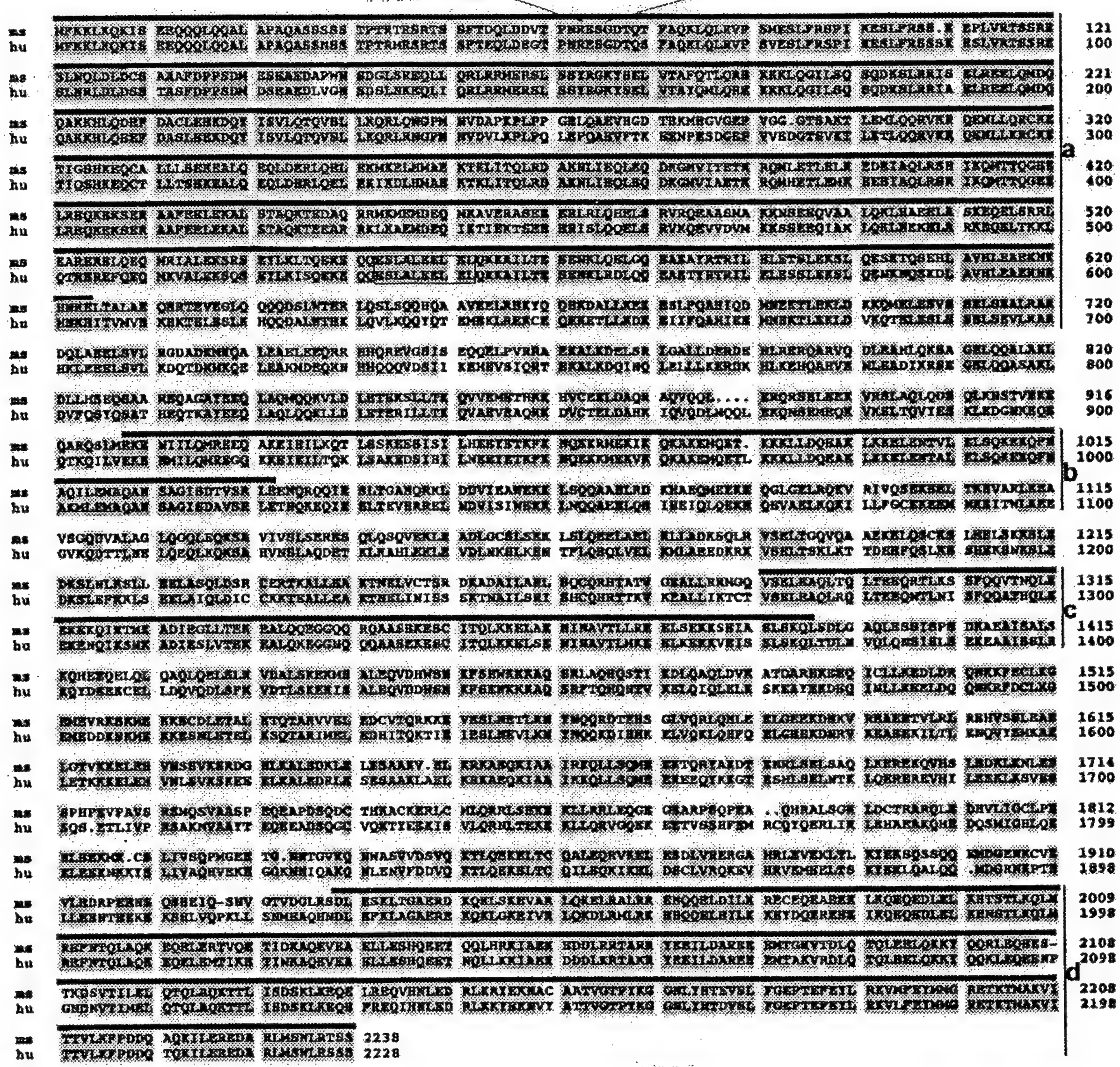
Expression of mouse tGolgin-1 mRNA: abundant expression in oligodendrocyte precursors

The distribution of human tGolgin-1 mRNA in different tissues has not been evaluated. To assess the tissue distribution of mouse tGolgin-1, RNA was prepared from a panel of mouse tissues and hybridized with a probe derived from the coding region of the mouse tGolgin-1 cDNA (Fig. 5). tGolgin-1 mRNA migrates as a single major band of approximately 7.5-kb in length, as expected from the length of the predicted full-length cDNA and by analogy with the human tGolgin-1 mRNA. Overexposure of the blot reveals additional bands from brain tissue at approximately 9- and 12.5-kb, which may represent incompletely spliced heteronuclear RNAs or alternative splice products; the 9-kb band was only observed in brain tissue (unpublished data). The 7.5-kb mRNA is expressed in all tissues analyzed, as expected for a gene product involved in constitutive transport processes. Relative to the GAPDH controls, no clear enrichment was observed in any tissue analyzed. Although these data do not rule out cell type variation in tGolgin-1 mRNA expression, they indicate that no tissue types are enriched in cells that express particularly high or low levels of tGolgin-1 mRNA.

Fig. 5

Because tGolgin-1 was identified using an antibody to OIP-1 expressed on oligodendrocyte precursors, we next sought to determine whether endogenous tGolgin-1 mRNA was more highly expressed specifically by this cell type. Sorted populations of neonatal mouse brain cells were analyzed by semi-quantitative RT-PCR. This assay utilized limited numbers of

A



B

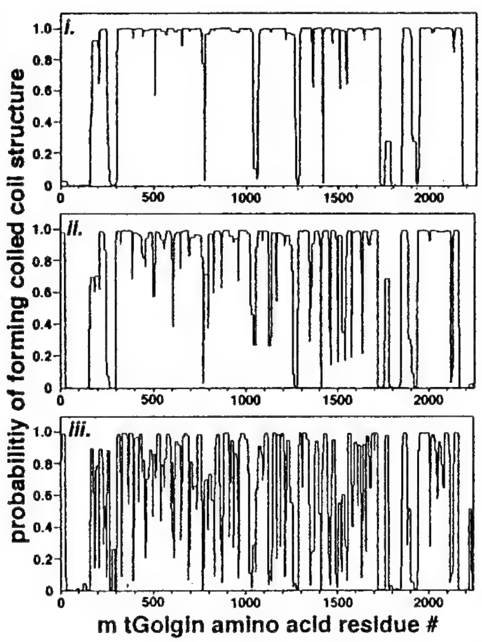


FIG. 2. Comparison of mouse and human tGolgin-1 amino acid sequences and coiled-coil predictions. (A) Deduced amino acid sequence of mouse (ms) tGolgin-1 and alignment with that of human (hu) tGolgin-1. The human tGolgin-1 sequence listed includes the N-terminal regions present in *trans*-golgi p230 and 256 kD golgin, but the C-terminal region of golgin-245 containing the region encoded by the 63bp alternatively spliced exon. Amino acid identities are darkly shaded and similarities are lightly shaded. (sentence) Potential granin motifs are boxed. (B) Coiled-coil predictions using the MTIDK matrix and windows of 14 (i), 21 (ii), and 28 (iii) residues without weighting of positions 1 and 4. Similar patterns were obtained with weighting of these positions and using the MTK matrix.

(ms) (hu)

sentence below

Regions of marked homology between mouse and human sequences are indicated by thick lines above the sequence and by the letters a, b, c and d to the right of the sequence.

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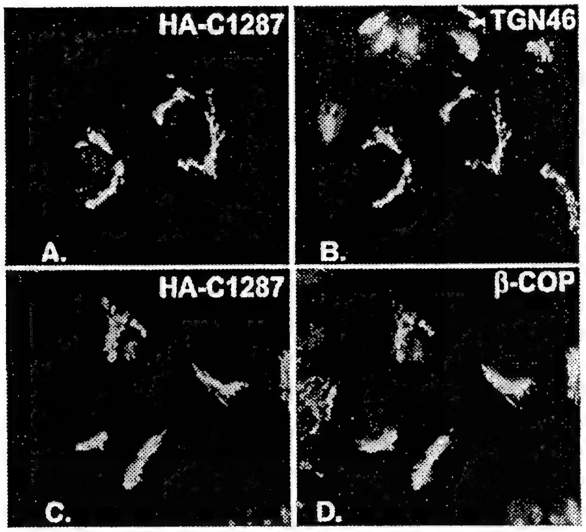


FIG. 3. The HA-tagged gene product of partial tGolgin-1 clone C91 localizes to the TGN region. (**A–D**) HA-C1247, representing the C-terminal 1247 residues of tGolgin-1 tagged at the N-terminus with the HA11 epitope, was expressed in HeLa cells by transient transfection. Cells were fixed and stained with antibodies to HA11 (**A, C**) and to either endogenous TGN46 (**B**) or endogenous β-COP (**D**) and FITC- and Rhodamine Red X-conjugated secondary antibodies. **A, B** and **C, D** show representative immunofluorescence microscopy staining patterns from identical fields of cells. Note the presence of both transfected and untransfected cells in the same fields.

PCR amplification cycles to assess the relative frequency of different mRNAs. First, oligodendrocyte precursors were sorted into three populations as defined by their expression of well-characterized developmental markers (see Fig. 6A and Gay *et al.*, 1997, for developmental analyses). Population 1 ($O4^+$ R-Mab^-) contains the least mature cells, which express the O4 epitope characteristic of committed oligodendrocyte precursors but not the Ranscht epitope (R-Mab) characteristic of more differentiated, less proliferative cells. Population 2 ($O4^+$R-Mab^+) contains cells at an intermediate stage of development in which R-Mab expression has been initiated but lack expression of myelin components. Population 3 (Gal-C+) contains the most mature cells that express myelin components; the first two populations contained no Gal C^+ cells, as determined by flow cytometry (unpublished data). mRNA from equal cell numbers of each population was subjected to semiquantitative PCR using various primer sets. Using primers corresponding to actin cDNA, a linear increase in accumulation of actin product was seen with increasing rounds of amplification, although both astrocytes and fibroblasts contained much higher amounts of product, most likely reflecting their larger cell size and increased mRNA levels per cell (Fig. 6A).

Fig. 6

RT-PCR products corresponding to DM20 and PLP, two temporally regulated proteins encoded by the same gene (Macklin *et al.*, 1987), were assessed to verify the precursor stages under study. During oligodendrocyte development, DM20 transcription precedes that of PLP (Ikenaka *et al.*, 1992; Timsit *et al.*, 1992). As predicted, the smaller DM20 product was found in population 1, whereas both the DM20 and PLP products were found in the more mature populations 2 and 3 (Fig. 6A). Astrocytes also expressed some DM20/PLP products, most likely reflecting a few mature oligodendrocytes in this unsorted cell culture. These data validate that our sorted populations were relatively pure and that the RT-PCR assay faithfully reproduced previous characterizations.

To analyze for the presence of tGolgin-1 transcripts, PCR was performed using primers corresponding to a region of the cDNA sequence present in C91 (see Fig. 1a for PCR product locations along the cDNA sequence). As can be seen in Figure 6A, a PCR product of the appropriate size was obtained from populations 2 and 3. Population 1, astrocytes, and fibroblasts contained very weak bands, which may correspond to low levels of tGolgin-1 transcripts (see below). These data indicate that tGolgin-1 mRNA is expressed most extensively by stage II precursors. This corresponds to the appropriate developmental window for OIP-1 expression, and is the first demonstration that transcription of tGolgin-1 may be differentially regulated.

The RT-PCR analyses were extended using oligodendrocyte precursor populations sorted for expression of OIP-1 and R-

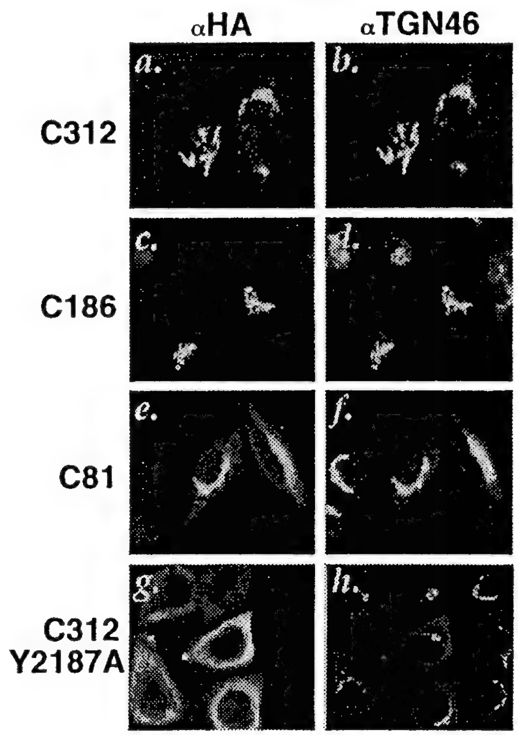


FIG. 4. GRIP domain-dependent localization of C-terminal tGolgin-1 fragments to the TGN. N-terminal HA11-epitope tagged truncations of tGolgin-1 were expressed in HeLa cells by transient transfection, and fixed cells were stained with antibodies to HA11 (**a, c, e, g**) and to endogenous TGN46 (**b, d, f, h**) and fluorophore-tagged secondary antibodies. Cells were analyzed by immunofluorescence microscopy using a conventional (**a–f**) or confocal scanning (**g, h**) microscope. The truncations comprised the C-terminal 312 (C312; **a, b**), 187 (C187; **c, d**), or 81 (C81; **e, f**) residues. In **g, h**, the cells expressed C312 with a mutation changing the conserved tyrosine at position 2187 to an alanine (C312.Y2187A).

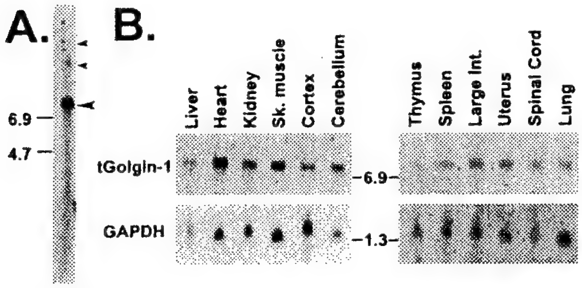


FIG. 5. Northern blot analysis of the tissue distribution of tGolgin-1 mRNA. Five micrograms of polyA+ RNA prepared from neonatal mouse brain (**A**) or 20 μg of total RNA from the indicated tissues (**B**) was fractionated on agarose gels and transferred to nitrocellulose. Blots were developed with digoxigenin-conjugated probes for tGolgin-1 or GAPDH, as indicated, followed by a luminescence detection system and phosphorimaging analysis. The position of RNA markers is indicated. In **A**, the large arrowhead points to the main band estimated at 7.5 kb; this band is also highlighted in the blots in **B**. Small arrowheads in **A** point to minor RNA species only detected in longer exposures, as discussed in the text.

Mab (Fig. 6B). OIP-1$^-$R-Mab$^-$ (least mature population 1), OIP-1$^+$R-MAb$^+$ (intermediate stage II population 2), and OIP-1$^-$R-Mab$^+$ (late stage II population 3) cells were subjected to identical conditions as in Figure 6A except that a second set of tGolgin-1 primers was also included for analyses. Again, only intermediate and late stage II cells generated PCR products corresponding to the tGolgin-1 sequence, and products correlated precisely with surface OIP-1 expression.

The Northern analyses described above suggested that tGolgin-1 is expressed in all tissues, and hence, in many cell types. To confirm that the RT-PCR assay could detect tGolgin-1 transcripts expressed at lower levels in cells other than oligodendrocyte precursors, two populations found negative by semi-quantitative PCR, astrocytes, and fibroblasts, were subjected to high cycle RT-PCR. At 40 cycles, weak PCR products were observed (Fig. 6C). These data support the view that the tGolgin-1 mRNA is expressed in many cells but is particularly abundant in oligodendrocyte precursors.

DISCUSSION

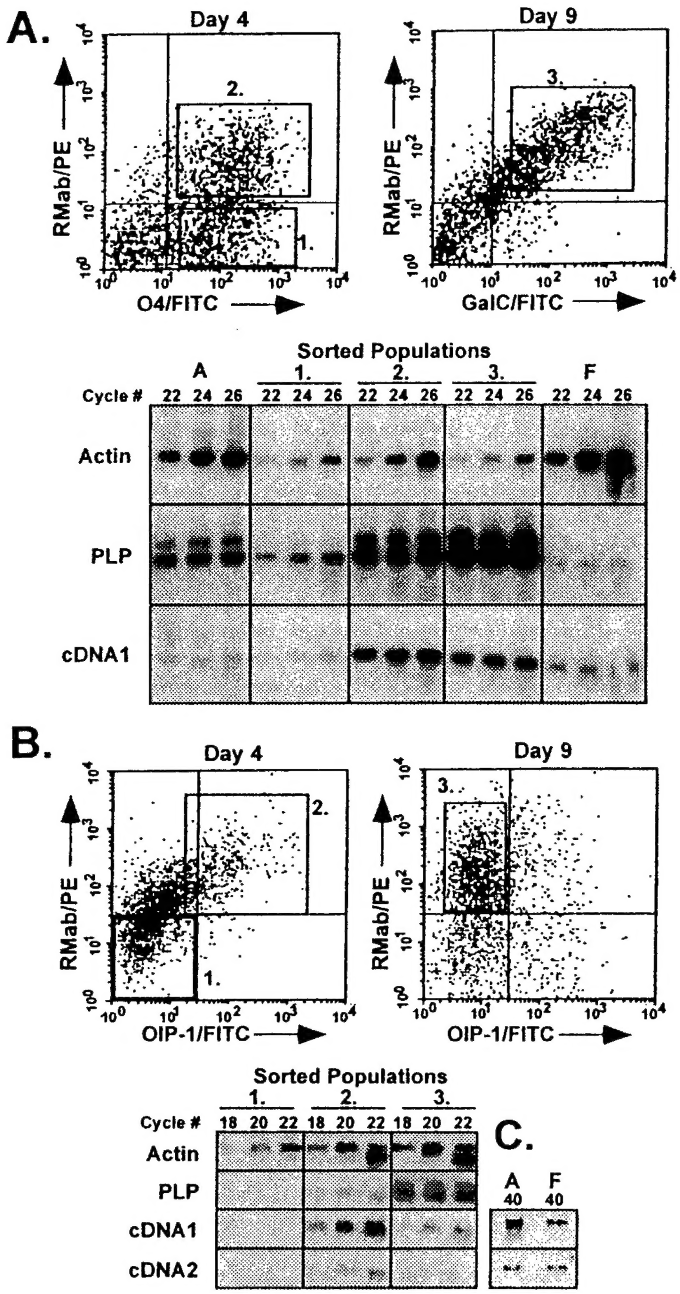
Identification of the murine tGolgin-1 homolog

Several groups identified and characterized a large, Golgi-localized protein as the target of autoantibodies in patients with Sjögren's Syndrome (Kooy *et al.*, 1992; Fritzler *et al.*, 1995). These groups cloned three homologous cDNAs that putatively encode proteins termed golgin-245 (Fritzler *et al.*, 1995), *trans*-golgi p230 (Erlich *et al.*, 1996), and 256 kD Golgin (submitted to Genbank by H. P. Seelig), all of which reacted with patient antisera, but which differed in sequence at the 5′ and 3′ end. The sequences of these three proteins are nearly identical throughout their coding region; careful analysis of the sequence data indicates that they represent partial or complete clones of the same cDNA, except for the inclusion or exclusion of a 63bp exon near the coding region for the extreme C-terminus. The extreme 5′ terminus of the golgin-245 sequence begins at the coding region for amino acid #130 of the *trans*-golgi p230 sequence, and we therefore believe that this is a 5′-truncated partial clone. The sequence of 256 kD golgin differs from that of *trans*-golgi p230 by the exclusion of a single G nucleotide at position 6666 within a poly-G tract; this shifts the reading frame for the predicted amino acid sequence to differ from those of *trans*-golgi p230 and golgin-245 near the C-terminus, and we therefore believe that this is a sequencing error. Because these proteins were localized to the *trans*-Golgi network and associated vesicles (Gleeson *et al.*, 1996), we refer to them collectively as tGolgin-1. Here, we identify the cDNA for the mouse homolog of tGolgin-1, and show that it is expressed in particularly great abundance in oligodendrocyte precursors.

We previously described a unique cell surface marker on stage II oligodendrocyte precursors that was referred to as OIP-1 (Gay *et al.*, 1997). Using the anti-OIP-1 mab to screen a postnatal mouse brain cDNA expression library, we isolated three overlapping cDNAs that encoded a protein with homology to the C-terminal half of human tGolgin-1. Using the mouse EST database and RT-PCR, we extended these cDNAs to span a 7530 bp region with homology to the entire human tGolgin-1 cDNA. Several data suggest that this clone represents the major or only mouse homolog of human tGolgin-1. First, the mouse tGolgin-1 clones exhibited strong homology to human tGolgin-1, and a similar splice variation of a 63 bp exon occurred at the extreme 3′ end of the coding region. Second, all of the available murine EST sequences with homology to human tGolgin-1 exhibited complete or nearly complete identity with the mouse sequence. These ESTs were derived from diverse embryonic and adult cDNA libraries. Third, like its human counterpart, HA-tagged truncated forms of the mouse protein localized to the TGN in transfected cells and could be partially displaced following treatment with brefeldin A. Finally, comparable to the analyses made of human tGolgin-1 thus far, the mouse mRNA was ubiquitously expressed, albeit at much higher levels in oligodendrocyte precursors. Collectively, this evidence suggests that we have identified the only mouse homolog of human tGolgin-1.

Interestingly, the degree of similarity between the human and mouse proteins varied throughout the coding region. Regions of highest homology (>90% identical) were present at the extreme C-terminus, spanning the GRIP domain and adjacent predicted coiled-coil regions, and within the 600 amino-terminal residues. These were interspersed with regions of modest homology (60–70% identity) throughout the bulk of the protein, within regions predicted to have a coiled-coil structure. We speculate that the less conserved regions retain general structural features that are required for tGolgin-1 function, such as coiled-coil structure, whereas the more highly conserved regions likely mediate specific interactions within a tGolgin-1 oligomer or with other conserved proteins or nonprotein effectors.

It is curious that three independent cDNA clones with overlapping sequences for tGolgin-1 were identified using an antibody to a cell surface markers ~~protein~~ on oligodendrocyte precursors. marker
Although we have as yet been unable to express the full-length mouse tGolgin-1 protein in transfected cells, no cell surface expression of the clone C91 gene product could be detected with



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ℓ mouse

FIG. 6. tGolgin-1 mRNA is highly expressed in oligodendrocyte precursors. Semiquantitative RT-PCR (**A**, **B**) of sorted oligodendrocyte precursor populations, astrocytes, and fibroblasts and 40-cycle RT-PCR (**C**) of astrocytes and fibroblasts. Top panels represent flow cytometric profiles of cultured day 4 (left) or day 9 (right) ~~rat~~ a mouse oligodendrocyte precursors colabeled with either anti-O4 or anti-GalC antibodies conjugated to FITC and PE-conjugated R-Mab as indicated. Sorted cells are boxed and numbered to indicate populations analyzed by RT-PCR (lower panels). Lower panels show PCR products from sorted populations, astrocytes, and fibroblasts using specific primers against designated proteins. Note that in **A**, PLP and cDNA1 PCR products were close to saturating levels even at the lowest cycle numbers (22, 24, 26 cycles). More representative samples, collected at lower cycle numbers (18, 20, 22 cycles), can be seen in **B**.

either anti-HA or anti-OIP-1 antibodies, and antibodies to human tGolgin-1 are nonreactive with the cell surface of human cell lines (unpublished data). Furthermore, except for the initial isolation from bacteriophage plaques of bacterial cultures, we were unable to demonstrate direct reactivity of the tGolgin-1 gene product with the anti-OIP-1 antibody using several assays (unpublished data). Finally, the deduced amino acid sequence contains no apparent signal sequence, consistent with the structure of a peripheral membrane protein. Nevertheless, endogenous tGolgin-1 mRNA levels were greatly upregulated in stage II oligodendrocyte precursors, compared with precursors at other developmental stages or unrelated cells such as astrocytes and fibroblasts; this is the same developmental stage at which OIP-1 surface expression is observed. We speculate that either the OIP-1 determinant represents a small amount of surface expression of tGolgin-1 in cells in which it is highly expressed, or that high levels of tGolgin-1 protein are required to generate a determinant on a distinct molecule that can be recognized by the anti-OIP-1 mab (see below). Surface expression of a normally cytoplasmic protein would be unusual, but not unprecedented (Cleves and Kelly, 1996). Indeed, cellular myosin II, a protein with some homology to tGolgin-1 that also participates in vesicular transport from the TGN (Musch *et al.*, 1997; Stow *et al.*, 1998), has been reported to associate tightly with the neuronal plasma membrane (Li *et al.*, 1994) and to localize to the cell surface (Michelis *et al.*, 1994; Yanase *et al.*, 1997).

A possible function for tGolgin-1 in the vesicular trafficking of glycolipids or proteolipids?

It has been postulated that components of the vesicular transport machinery are upregulated to accommodate myelination. Indeed, Rab3a and Rab3c, GTP-binding proteins associated with regulated secretion in neurons and other specialized cell types, have been shown to be upregulated during the late development and maturation of oligodendrocytes (Madison *et al.*, 1996). Oligodendrocytes have been shown to possess features of polarized sorting (de Vries *et al.*, 1998), and express a unique Rab protein, Rab22b, which is thought to be involved in traffic between the TGN and endosomes (Rodriguez-Gabin *et al.*, 2001). We found elevated tGolgin-1 mRNA levels at a point in development slightly earlier than that in which myelin deposits accumulate, that immediately preceding and coincident with high surface expression of R-Mab antigen. These results suggest that expression of tGolgin-1 can be upregulated to accommodate specific vesicular transport functions in oligodendrocyte precursors, such as an increased cargo load or specialized cargo. A role in the transport of specialized cargo is supported by the finding that human tGolgin-1 and another TGN-associated peripheral membrane protein, myosin II, are present on separate vesicles associated with the TGN (Gleeson *et al.*, 1996; Brown *et al.*, 2001).

What might this specialized cargo be? Given the unique role of glycosphingolipids in oligodendrocyte biology, we speculate that tGolgin-1 may be involved in transport or synthesis of glycolipids. Glycolipids, including sulfatides such as the R-Mab antigen, are highly enriched in stage II oligodendrocyte precursors (Bansal *et al.*, 1989; Bansal and Pfeiffer, 1992) at the same stage in which we find high levels of tGolgin-1 mRNA. Glycolipids are manufactured and assembled into myelin at the TGN (Simons *et al.*, 2000) and progress to the cell surface through vesicular transport (Burkart *et al.*, 1982; Gow *et al.*, 1994). Glycosphingolipids play a general role in regulating Golgi export (Sprong *et al.*, 2001b), protein sorting processes at the TGN (van Meer, 1998; Sprong *et al.*, 2001a), and retrograde transport from the plasma membrane to the TGN (Falguières *et al.*, 2001; Johannes and Goud, 1998). Moreover, the differentiation of oligodendrocytes has been shown to be associated with distinct glycolipid sorting processes (Watanabe *et al.*, 1999), and maintenance of oligodendrocyte processes requires glycolipid cycling between the plasma membrane and the TGN (Benjamins and Nedelkoska, 1994). We speculate that tGolgin-1 may be involved in either the transport of glycolipids or sulfatides such as the R-Mab antigen (Bansal *et al.*, 1989; Bansal and Pfeiffer, 1992), or in the localization of resident TGN enzymes that facilitate glycolipid or sulfatide synthesis. Perhaps the anti-OIP-1 antibody recognizes a glycolipid that binds to tGolgin-1 and for which cell surface expression or recycling is facilitated by tGolgin-1.

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AU1
location? Noted - Indianapolis, IN

QU2
Figure 1 layout ok? Yes! Lovely!

Au: Degree?